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In Vivo RNA Interference Screening Identifies a Leukemia-Specific Dependence on Integrin Beta 3 Signaling

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SUMMARY

We used an *in vivo* short hairpin RNA (shRNA) screening approach to identify genes that are essential for MLL-AF9 acute myeloid leukemia (AML). We found that Integrin Beta 3 (*Itgb3*) is essential for murine leukemia cells *in vivo*, and for human leukemia cells in xenotransplantation studies. In leukemia cells, *Itgb3* knockdown impaired homing, downregulated LSC transcriptional programs, and induced differentiation via the intracellular kinase, Syk. In contrast, loss of *Itgb3* in normal HSPCs did not affect engraftment, reconstitution, or differentiation. Finally, we confirmed that *Itgb3* is dispensable for normal hematopoiesis and required for leukemogenesis using an *Itgb3* knockout mouse model. Our results establish the significance of the *Itgb3* signaling pathway as a potential therapeutic target in AML.

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by increased proliferation and impaired differentiation of hematopoietic stem and progenitor cells (HSPCs). With current treatments, the 5-year overall survival in adult AML is less than 20% and has improved only modestly in the past 30 years (Maynadie et al., 2011). The development of novel therapies with greater efficacy and decreased toxicity requires the identification of specific dependencies in leukemia cells that are absent in normal HSPCs *in vivo* (Gilliland et al., 2004).

Leukemia stem cells (LSCs) are a self-renewing subpopulation capable of initiating the disease upon transplantation into healthy recipients (Lapidot et al., 1994). LSCs reside in an *in vivo* microenvironment, as do normal HSPCs (Scadden, 2007). Increasing evidence indicates that the niche for malignant cells can influence disease initiation (Raaijmakers et al., 2010), lineage decisions (Wei et al., 2008), cellular localization, and response to chemotherapy (Ishikawa et al., 2007). Successful strategies to target the interaction of LSCs with the microenvironment using both small molecules (Parameswaran et al., 2011; Zeng et al., 2009) and biologics (Chao et al., 2010; Jin et al., 2006) have been reported.

Monotypic cell culture lines used in some traditional high-throughput drug discovery efforts may not reflect the primary disease from which they were derived (Drexler et al., 2000; Sharma et al., 2010) and may therefore be unable to probe important interactions between primary leukemia cells and the hematopoietic niche, or to identify which of these interactions are selectively required for leukemia cells relative to normal HSPCs. Indeed a number of “non-traditional” screens have identified important modulators of disease biology (Guzman et al., 2005; North et al., 2007; Yeh et al., 2009).

Pooled *in vivo* RNA interference (RNAi) screens offer a strategy to identify novel therapeutic targets for leukemia in their physiologic microenvironment. In this approach, primary leukemia cells enriched for stem cell activity, capable of generating leukemia in mice, are infected with a pool of lentiviruses expressing short hairpin RNAs (shRNAs). Transduced cells are transplanted into recipient mice where they engraft and grow in the host microenvironment. The quantitative representation of each shRNA in the pool of cells prior to transplantation and at subsequent time points can be determined using massively parallel sequencing, highlighting genes that are essential for malignant cells (Luo et al., 2008; Mendes-Pereira et al., 2011).

We employed this approach to find therapeutic targets in primary murine and human AML cells using the *MLL-AF9* mouse model of the human leukemia. A number of *MLL* translocations, including *MLL-AF9* and *MLL-ENL*, have been shown in mouse models to transform committed hematopoietic progenitors (Cozzio et al., 2003; Krivtsov et al., 2006). We performed a series of *in vivo* shRNA screens to identify and genes selectively essential for leukemia cells compared to normal HSPCs, and to explore downstream signaling molecules.

RESULTS

Pooled *In Vivo* shRNA Screening of Primary Murine Leukemia

To achieve a robust pooled *in vivo* shRNA screen in primary AML cells, we sought to use a model with labeled leukemia cells, an established cell surface marker phenotype of leukemia stem cells, a short latency, and a high penetrance. To this end, we introduced the *MLL-AF9* oncogene via retroviral transduction into flow-sorted granulocyte-monocyte progenitor cells (GMPs) from Actin-dsRed transgenic mice, enabling rapid identification of leukemic cells within wild-type tissue. Previous studies have shown that expression of *MLL-AF9* in normal GMPs is sufficient to create an aggressive, transplantable myeloid leukemia with functionally defined LSCs that display an immunophenotype similar to normal GMPs (Lin^{lo}, Sca-1, c-Kit^{+}, Fc$\gamma$$RII^{hi}$, $CD34^{hi}$) (Krivtsov et al., 2006). We further enriched for stem cell activity by serially transplanting the leukemias through secondary, tertiary, and quaternary recipients, generating fluorescently labeled leukemias with 100% penetrance (Figure S1A). We noted predictable and reproducible engraftment of leukemia cells in the bone marrow and spleen of recipient mice after transplant (Figure S1B).

We performed a primary screen using a pool of lentiviruses, each of which expressed one of 1352 shRNAs, targeting 268 genes plus 66 control shRNAs that are not homologous to the sequence of any murine genes (Figure 1A). The targets include known and candidate cancer-associated genes and genes that were discovered in previous unbiased RNA interference screens (Tables S1). Sorted leukemia cells (Figure 1B) from quaternary transplant MLL-AF9-dsRed mice were transduced with the lentiviral shRNA pool and transplanted into sublethally irradiated recipients. In addition, an aliquot of leukemia cells was plated onto OP9 stromal cells, a well-established murine, bone marrow-derived stromal cell line capable of supporting primary hematopoietic stem and progenitor cells for many weeks *in vitro* in the absence of cytokine supplementation (Nakano et al., 1994). Using massively parallel sequencing of PCR-amplified shRNA sequences from genomic DNA, we quantified the relative representation of each shRNA in the infected leukemia cells immediately after infection, and in cells harvested 2 weeks later from the bone marrow, spleen, and *in vitro* culture. We selected 60 candidate genes targeted by at least two shRNAs that were most highly depleted over 2 weeks (candidate genes that are required for leukemia cells), and 15 genes targeted by at least two shRNAs that were most increased over two weeks *in vivo* or *in vitro*.

We systematically re-tested all 75 candidate genes in an *in vivo* validation screen (Figure 1C), achieving 20-fold higher coverage of leukemia cells per shRNA than in the primary screen and high inter-replicate reproducibility (Figures 1D, S1C–G). The relative representations of each shRNA after 2 weeks in the bone marrow and spleen were highly correlated ($R^2 = 0.85$, Figure 1C), demonstrating the reproducibility of the screen and the biological similarity of leukemia cells in these organs. The top hits demonstrate that the shRNA screen highlights biologically relevant molecules (Figure 1E). Consistent with the recently reported requirement of β-catenin in MLL-AF9 leukemia stem cells (Wang et al., 2010; Yeung et al., 2010), all three shRNAs targeting β-catenin were depleted greater than 20-fold (Figure 1F) and shRNAs targeting *Apc*, a negative regulator of β-catenin activity,

were enriched in leukemia cells after two weeks (Figure 1G and S1H). At least two shRNAs per gene were depleted for additional genes known to be required for MLL-AF9 leukemia *(Mef2c, Ccna1)* (Ekberg et al., 2005; Krivtsov et al., 2006; Liao et al., 2001); genes universally required for cell survival *(Ube2j2, Utp18)* (Luo et al., 2008); genes reported to be essential in other AML models *(Hmgb3)* (Petit et al., 2010; Somervaille et al., 2009; Wang et al., 2005); and *Myb*, a gene important for LSC and HSPC survival (Lieu and Reddy, 2009; Somervaille et al., 2009). The performance of shRNAs targeting these genes demonstrates that our *in vivo* screen is capable of detecting the activity of genes with biological relevance for MLL-AF9 leukemia cells.

Integrin Beta 3 is Essential for Leukemia Cells

The top 3 hits from the validation screen were two positive controls with established importance in MLL-AF9 leukemia, *Ctnnb1* and *Hmgb3* (Somervaille et al., 2009; Wang et al., 2010), and *Itgb3*, encoding Integrin Beta 3. In all cases, three independent shRNAs were depleted by more than 20-fold over 2 weeks (Figures 1F and 1H and Table S2). Integrins mediate many cellular processes and interact with multiple components of the bone marrow including fibronectin, vitronectin, osteopontin, and bone sialoprotein, raising the possibility that Itgb3 plays a role in the interaction of leukemia cells with the microenvironment (Seiffert, 1996; Stier et al., 2005; Zhang et al., 2009). The previously reported *Itgb3* germline knockout mouse has normal peripheral blood counts, aside from platelet defects resulting from disruption of the Itgb3/Itga2b (GPIIb/IIIa) receptor (Hodivala-Dilke et al., 1999) and the conditional knockout animal has no hematopoietic defect in the primary recipient with a mild defect in reconstitution in the secondary recipient (Umemoto et al., 2012). Additionally, Glanzmann's Thrombasthenia is a human disorder of impaired platelet activation, resulting from mutations in either *ITGB3* or *ITGA2b* which together form the GPIIb/IIIa receptor on platelets (Nurden et al., 2011). Critically, aside from the platelet activation defect, patients with biallelic *ITGB3* mutations do not have a bone marrow failure phenotype, highlighting the potential dispensability of *ITGB3* in long-term hematopoietic stem cell maintenance and function.

We first confirmed the functional effects of *Itgb3* and *Ctnnb1* shRNAs. We verified that primary murine leukemia cell express Itgb3, and that all three shRNAs targeting *Itgb3* and *Ctnnb1* effectively decreased expression of their target gene (Figures 2A and S2A–B). To track the shRNA-carrying leukemia cells by flow cytometry, we inserted the *Ctnnb1, Itgb3*, and control *(shLuc)* shRNAs into a lentiviral vector that co-expresses GFP. We transduced leukemia cells with these shRNA-lentiviruses expressing GFP, transplanted them into sublethally irradiated recipients, and followed the proportion of GFP^+ leukemia cells over time (Figure S2C). In contrast to the pooled screening approach, each mouse was transplanted with leukemia cells transduced with lentivirus expressing a single shRNA. Consistent with the results of the pooled screen, the proportion of leukemia cells expressing *Ctnnb1* and *Itgb3* decreased dramatically over 14 days *in vivo*, while the percentage of leukemia cells expressing the control shRNA was stable in the bone marrow (Figures 2B–C) and spleen (Figures S2D–E). We also found that after 7 days of growth *in vivo*, leukemia cells carrying *Itgb3* shRNAs continued to have decreased cell surface expression of Itgb3 (Figure S2F).

Of note, despite homing and engrafting in the bone marrow at day 6, the disease burden of leukemia cells carrying *Itgb3* shRNAs progressively declines over time, reflecting the continued activity of the shRNAs in cells in the niche. To evaluate the possibility that the observed phenotype was solely from impaired homing of the cells to the bone marrow, we assessed the activity of the *Itgb3* shRNAs in an *ex vivo* assay. Primary leukemia cells were harvested, infected with *Itgb3* or control shRNAs then grown in isolation with murine IL-3 or in co-culture with OP9 cells, murine bone marrow stromal cells previously been shown to

support *ex vivo* growth of HSPCs (Nakano et al., 1994), and capable of supporting primary leukemia cells in the absence of cytokine supplementation. Regardless of culturing condition, the leukemia cells carrying *Itgb3* shRNAs were depleted over time, reflecting that these shRNAs can act in a homing-independent fashion (Figure S2G). Importantly, we did not observe any appreciable effect of lentiviral infection on cell surface Itgb3 expression (Figure S2H).

As any individual shRNA might produce off-target effects that influence the observed phenotypes, we confirmed the specificity of the *Itgb3* shRNAs. We designed an *Itgb3* cDNA (*Itgb3*$_{Rescue}$) with at least 6 silent mutations at each of the shRNA binding sites and inserted the cDNA into the pMSCV-IRES-GFP (pMIG) retroviral backbone. Expression of pMIG-*Itgb3*$_{Rescue}$ resulted in high cell expression of Itgb3 on primary leukemia cells *in vivo* and did not significantly alter the growth characteristics of the leukemia cells after 2 weeks in either the bone marrow or spleen (Figures S2I–J). In Ba/F3 cells, introduction of pMIG-*Itgb3*$_{Rescue}$ resulted in high levels of Itgb3 expression that was maintained despite expression of *Itgb3* shRNAs (Figure S2K). Leukemia cells were serially transduced with pMIG-*Itgb3*$_{Rescue}$ and either *Itgb3* or control shRNAs co-expressing the puromycin resistance gene, selected in puromycin for 2 days, and transplanted into recipient mice (Figure S2L). GFP^{+} cells carrying the *Itgb3*$_{Rescue}$ vector increased in the leukemias co-expressing the *Itgb3* shRNAs, but not in the leukemias expressing a control shRNA over two weeks *in vivo* (Figure 2D), reflecting the ability of *Itgb3*$_{Rescue}$ to abrogate the effects of the *Itgb3* shRNAs. Confirming our prior results, the lack of GFP^{+} cell expansion in the *Itgb3*$_{Rescue}$ plus shLuc control demonstrates that *Itgb3*$_{Rescue}$ alone does not change the growth properties of the leukemia cells.

We next sought to determine whether knockdown of *Itgb3* would confer a survival advantage to recipient mice. Sublethally irradiated recipient mice were transplanted with 50,000 leukemia cells, all of which expressed *Itgb3* shRNAs, in contrast to the GFP tracking experiment in which only a subset of cells expressed GFP (Figure S2C). Compared to the control, mice transplanted with leukemia cells expressing *Itgb3* shRNAs lived significantly longer (Figure 2E). These experiments demonstrate that *Itgb3* shRNAs effectively impair leukemia growth *in vivo*.

The Heterodimer Itgb3/Itgav Is Required for Leukemia Cell Survival

As Itgb3 heterodimerizes with Itgav in myeloid cells (Savill et al., 1990), we hypothesized that knockdown of *Itgav* would phenocopy knockdown of *Itgb3*, providing further evidence of the function of Itgb3 in leukemia. We first confirmed the co-expression of Itgav and Itgb3 on leukemia cells in our model by flow cytometry (Figures 3A and S3A). Consistent with the known interaction of Itgb3 and Itgav, knockdown of *Itgb3* with shRNA *Itgb3*-20 decreased Itgav cell surface expression after 7 days *in vivo* (Figure 3B). Furthermore, using our GFP tracking approach, we found that leukemia cells expressing either of two shRNAs that suppress *Itgav* (Figure 3C) were depleted from the bone marrow and spleen after two weeks growth *in vivo* relative to a control shRNA, demonstrating that *Itgav*, like *Itgb3*, is required for leukemia cells (Figures 3D and S3B).

ITGB3 is a Target in Human Leukemia

To establish the relevance of ITGB3 to human disease, we examined the expression and functional importance of ITGB3 in human AML. Using flow cytometry we found examples of ITGB3/ITGAV expression on primary human samples of both MLL-rearranged and non-MLL-rearranged AML (Figure S3C). Next, we assessed the functional importance of human *ITGB3* in M9 cells, leukemia cells derived from umbilical cord blood cells transduced with the MLL-ENL oncogene (Barabe et al., 2007). M9 cells can either be passaged *in vitro* or

transplanted into immunodeficient mice, resulting in leukemia. We generated a pool of lentiviral shRNAs targeting the human counterparts of the genes that scored in our murine screen. Following transduction with the pooled lentivirus, M9 cells were transplanted into immunodeficient NOD-SCID/IL2R$\gamma^{-/-}$ (NSG) recipient mice and harvested after 21 days. The representation of shRNAs in the bone and spleen was assessed by massively parallel sequencing. As with the murine screens, the data were highly reproducible between replicates and between the bone marrow and spleen (Figure S3D). Both of the validated *ITGB3* shRNAs tested were depleted by 20-fold in the spleens and bone marrow of mice transplanted with M9 cells relative to control shRNAs, replicating the results from our murine screen in human cells with an independent set of shRNAs (Figure 3E).

To evaluate the functional relevance of ITGB3 for primary human disease, we performed xenotransplantation studies with primary human AML samples expressing *ITGB3* or control shRNAs. We examined two primary AML samples, one from a patient with cytogenetically normal AML and one from a patient with MLL-rearranged AML. We transduced a pool of lentiviruses with human *ITGB3* and control shRNAs into the primary leukemia cells. After 24 hours, half of the cells were harvested for processing and the remaining were transplanted into immunodeficient NSG mice. We monitored the mice for engraftment and progression of disease by peripheral blood hCD45 analysis. Bone marrow and spleens were harvested 6 weeks after transplant for the MLL-rearranged AML and 8 weeks for the cytogenetically normal AML. The representation of shRNAs was assessed by massively parallel sequencing. We found that two independent *ITGB3* shRNAs, both of which effectively decrease *ITGB3* expression, impaired primary leukemia cell growth of both samples by at least 10-fold in the bone marrow and spleens of recipient mice (Figures 3F and S3E). In aggregate, these findings demonstrate that, at least for a subset of primary human leukemia, ITGB3 is expressed on the cell surface and is functionally essential *in vivo*.

Knockdown of *Itgb3* Does Not Impair Normal HSPC Function *In Vivo*

The therapeutic opportunity for targeting *Itgb3* in leukemia depends on the selective importance of *Itgb3* for leukemia cells relative to normal HSPCs. In wild-type mice, we found Itgb3 expression on stem, progenitor, and mature myeloid cells. Expression was higher in stem cells (Lin^{lo} Sca-1^{+} c-Kit^{+} CD48^{lo} − LSK CD48^{lo}) compared to progenitor cells (LSK CD48^{hi}) (Figures 4A and S4A). Among progenitor cells, expression was highest in GMPs, which share the immunophenotype of the leukemia stem cell population in the MLL-AF9 model (Figure 4B) (Krivtsov et al., 2006). Mature myeloid cells (Mac1^{+} Gr1^{+}) had higher expression than immature myeloid cells (Mac1^{-} Gr1^{-}) (Figure S4B).

We examined the functional effect of acute *Itgb3* loss by transducing murine LSK cells with *Itgb3* or control shRNAs (Figure 4C and S4C) and transplanting the cells into lethally irradiated recipients. Since the infection efficiency was not 100%, only a portion of the LSKs expressed the shRNAs (and thus were GFP^{+}); thus the experimental setup represents a competitive transplant in which, within each mouse, the LSKs carrying an shRNA competed against the LSK that did not carry an shRNA. We found no significant difference between normal cells expressing *Itgb3* or control shRNAs, based on the percentage of GFP^{+} peripheral blood cells over 24 weeks. This finding stands in striking contrast to the profound *in vivo* growth inhibition mediated by the same *Itgb3* shRNA in leukemia cells (Figures 2C and 2E). We also performed peripheral blood lineage analysis at 24 weeks to determine if the *Itgb3* shRNA had any effect on differentiation. We found there was no significant difference in the levels of T, B, or myeloid cells between the two groups (Figure 4D) suggesting that *Itgb3* knockdown does not impair HSPC differentiation in the transplant model.

Itgb3 Knockdown Impairs Leukemia Cell Homing and Induces Differentiation

Given the role of integrins in multiple cellular processes, including proliferation and homing, we sought to determine the mechanism of leukemia cell depletion following *Itgb3* knockdown. First, using Hoechst staining, we found no differences in the cell cycle states of primary leukemia cells expressing *Itgb3-17*, *Itgb3-20*, or control shRNAs after 4 days (Figure S5A). We next examined whether decreased expression of Itgb3 alters primary leukemia cell homing. Primary leukemia cells were infected with *Itgb3* or control shRNAs, transplanted into sublethally irradiated recipients, and harvested from the long bones of mice after 16 hours. We found no difference in the representation of shRNA-carrying leukemia cells between the two groups (Figure S5B), implying that *Itgb3* knockdown does not cause a gross defect in bone marrow localization after transplant. Next, we used live, *in vivo* imaging of the murine calvarial bone marrow (Fujisaki et al., 2011) to investigate the possibility that, despite the ability of cells to travel to the bone marrow after *Itgb3* knockdown, there may be a defect in homing to the endosteum. Leukemia cells transduced either *Itgb3-20* or control shRNAs were isolated, stained with DiD, a membrane-bound fluorescent dye, and transplanted into lethally irradiated recipient mice. After 24 hours, identical volumes of calvarial bone marrow were imaged using intra-vital two-photon confocal microscopy. Compared to leukemia cells transduced with control shRNA, significantly fewer leukemic cells transduced with the *Itgb3-20* shRNA were anchored near the endosteal surface (Figures 5A and S5C) demonstrating that Itgb3 contributes to homing of leukemia cells *in vivo*.

We next examined the effect of *Itgb3* knockdown by gene expression profiling. Strikingly, 4 of the top 20 most upregulated probe sets in the Itgb3 knockdown samples encoded myeloperoxidase (MPO) and lysozyme (LYZ), two highly specific markers for myeloid maturation (Figure 5B). Gene set enrichment analysis (GSEA) (Subramanian et al., 2005) confirmed that, compared to a control shRNA, the *Itgb3* knockdown samples had significantly higher expression of a series of published myeloid differentiation signatures (Hahn et al., 2009; Krivtsov et al., 2006; Novershtern et al., 2011) and lower expression of a series of LSC signatures (Gal et al., 2006; Somervaille et al., 2009) (Figure 5C, Table S3).

In agreement with the GSEA results, compared to a control shRNA, leukemia cells expressing *Itgb3* shRNAs had significantly lower levels of c-Kit (Figure 5D and S5D), a cell surface protein that marks populations most enriched for LSCs (Krivtsov et al., 2006). Furthermore, histopathological analysis of cellular morphology following *Itgb3* knockdown revealed darker nuclei, decreased nuclear: cytoplasmic ratios, and increased monocytic morphologic changes, consistent with myeloid differentiation (Figure 5E). In contrast to the myeloid differentiation and LSC signatures, *Itgb3* knockdown did not alter three previously published β-Catenin gene signatures (Bild et al., 2006; Liberzon et al., 2011; Onder et al., 2008) (Figure S5E).

We then examined the activity of human *ITGB3* shRNAs in two human AML cell lines, HL-60 and U937, canonical models of differentiation therapy in AML. After confirming expression of ITGB3 and activity of *ITGB3* shRNAs in both cell types (Figure 5F), we found that *ITGB3* knockdown induced morphological evidence of differentiation (Figure 5G) and increased expression of a gene expression signature of myeloid differentiation, comparable to the effects of all trans-retinoic acid (ATRA), detected by multiplexed ligation-mediated amplification and detection of amplicons on fluorescent beads as previously described (Hahn et al., 2009) (Figures 5H and S5F). Both *ITGB3* shRNAs also dramatically increased cell surface expression of the mature myeloid markers CD14 and CD11b in HL-60 and U937 cells (Figures 5I and S5G), consistent with myeloid differentiation.

Identification of Mediators of *Itgb3* signaling in Leukemia

Having established that *Itgb3* knockdown selectively targets leukemia cells, impairs homing, and induces differentiation, we sought to determine whether mediators of Itgb3 signaling are also essential for MLL-AF9 transformed cells. Itgb3 signaling has been studied in platelets and other cell types, but not in leukemia (Kasirer-Friede et al., 2004; Kim et al., 2009). We sought to identify critical mediators of Itgb3 signaling in our primary mouse model using a comprehensive functional approach. We therefore systematically analyzed known pathway members with an additional pooled *in vivo* shRNA screen (Figures 6A and S6A) focused on 19 genes (97 shRNAs) with 7 control shRNAs to maximize the sensitivity of the *in vivo* readout. As expected, all of the shRNAs targeting *Itgb3* were powerfully depleted over 2 weeks. In addition, the screen highlighted multiple key mediators of Itgb3 signaling that are also essential for leukemia cells *in vivo* (Figures 6B and S6B). At least two validated shRNAs were depleted by greater than 10-fold over two weeks *in vivo* for the *Syk*, *Vav1*, *Rac2*, *Rhoa*, and *CD47* genes. Interestingly, nearly all of these hits have been previously implicated in hematologic malignancies, but their activity had not been linked to integrin signaling in this context.

We focused on *Syk* for three reasons. First, it is directly downstream of Itgb3 in megakaryocytes and therefore may be directly related to Itgb3 function in leukemia cells. Second, data from germline and conditional knockout animals suggest that in hematopoiesis, *Syk*, while potentially important for the function of select differentiated cells, may be dispensable for stem cell function (Cornall et al., 2000; Mocsai et al., 2002; Wex et al., 2011). Finally, multiple, large-scale phase II clinical trials of the small molecule SYK inhibitor, R406, the active metabolite of the soluble orally available SYK inhibitor fostamatinib (Braselmann et al., 2006), for idiopathic thrombocytopenic purpura and rheumatoid arthritis did not result in significant neutropenia among the treatment groups (Bajpai, 2009; Podolanczuk et al., 2009; Weinblatt et al., 2008). Thus, Syk may, in part, explain the selectivity of Itgb3 inhibition on leukemia cells.

We first demonstrated that individual, validated *Syk* shRNAs in GFP-expressing vectors impair leukemia growth in our model *in vivo* (Figures 6C and S6C–D). To examine whether Syk is an essential mediator of Itgb3 activity, we tested whether TEL-SYK, a constitutively active form of Syk (Kanie et al., 2004), could rescue the effect of *Itgb3* knockdown. Primary murine leukemia cells were serially transduced with pMIG-TEL-SYK and either *Itgb3-20* or control shRNAs (carrying the puromycin resistance gene). Transduced cells were selected with puromycin for 2 days, yielding cells that uniformly express the shRNA. A subset of this cell population also expresses TEL-SYK. Cells were then transplanted into sublethally irradiated recipients (Figure S6E). After two weeks, the proportion of GFP^+ ($TEL\text{-}SYK^+$) leukemia cells was significantly higher in the leukemias co-expressing the *Itgb3* shRNA compared to leukemias co-expressing the control shRNA (Figure 6D). Importantly, as evidenced by the absence of GFP^+ cell expansion in the TEL-SYK plus shLuc control, TEL-SYK alone does not change the growth properties of the leukemia cells *in vivo*. This result reflects the ability of the TEL-SYK fusion to rescue the inhibitory effects of *Itgb3* knockdown.

To further confirm the role of Syk in our model, we generated a gene expression signature comprised of genes that are upregulated in human leukemia cell lines after treatment with the Syk inhibitor, R406. Using gene set enrichment analysis, we found that, compared to a control shRNA, primary leukemia cells carrying the *Itgb3* shRNAs significantly increased expression of the R406 treatment signature (Figure 6E). We then tested the activity of R406 on leukemia cells from our model using a co-culture assay. OP9 mouse bone marrow stromal cells were plated in a clear-bottom 384-well plate and 24 hours later primary leukemia cells were added to the stromal layer. After 24 hours, R406 or XK469, a

topoisomerase II inhibitor and positive control for cell death (Kakodkar et al., 2011), were added to the co-culture at six doses. After six days, the plates were imaged and dsRed cells were counted. Mirroring the expected results with XK649, R406 inhibited leukemia cell growth in a dose-dependent fashion (Figure 6F).

Next, we examined the biochemical connection between ITGB3 and SYK. To establish the connection in the context of the MLL-AF9 translocation, we used the Mono-Mac-6 and MOLM-13 cell lines, both of which carry the MLL-AF9 translocation (Matsuo et al., 1997; Ziegler-Heitbrock et al., 1988). The cell lines were transduced with two human *ITGB3* shRNAs, selected in puromycin, and assessed for levels of phosphorylated SYK (p-SYK). In both cell lines knockdown of *ITGB3* resulted in a significant reduction of p-SYK as measured by intracellular phospho-flow (Figures 6G–H and S6F–G). Western blot analysis confirmed that knockdown of ITGB3 reduced levels of p-SYK without changing overall SYK levels in both cell types (Figures 6I and S6H). To extend the results beyond MLL-AF9, we transduced SKM1 cells, originally isolated from a patient with myelomonocytic leukemia (Nakagawa and Matozaki, 1995), with *ITGB3* shRNAs. Western blot analysis again revealed that both *ITGB3* shRNAs caused a powerful decrease in phospho-SYK (p-SYK) levels without changing overall SYK levels (Figure S6I), again demonstrating that ITGB3 directly impacts SYK activation in leukemia cells.

Germline Loss of *Itgb3* Selectively Impairs Leukemogenesis

Finally, to confirm the selective role of *Itgb3* in leukemia cells relative to normal HSPCs, we examined a definitive genetic model with homozygous germline inactivation of *Itgb3*. In agreement with the normal peripheral counts previously reported for the *Itgb3* knockout mice (Hodivala-Dilke et al., 1999), we found that there was no difference in the absolute number or percentage of hematopoietic progenitors in the bone marrow of $Itgb3^{-/-}$ and $Itgb3^{+/-}$ groups (Figures 7A and S7A–B). Next, we sought to characterize the effect of germline *Itgb3* loss on HSPCs using long-term competitive reconstitution assays. One million bone marrow cells from either $Itgb3^{-/-}$ or littermate control $Itgb3^{+/+}$ mice (CD45.2) were transplanted along with one million wild type competitor bone marrow cells (CD45.1/2) into lethally irradiated recipient mice (CD45.1) (Figure S7C). We assessed the chimerism of the hematopoietic compartment over 22 weeks by comparing CD45.1, CD45.1/2, and CD45.2 levels in the peripheral blood. There was no difference in the levels of chimerism between $Itgb3^{-/-}$ and $Itgb3^{+/+}$ bone marrow (Figure 7B) indicating that germline loss of *Itgb3* does not impair the ability of normal HSPCs to home, engraft, or grow in a long term *in vivo* transplantation assay. Finally, we analyzed the distribution of differentiated cells in recipient mice that received either $Itgb3^{-/-}$ and $Itgb3^{+/+}$ bone marrow and found that peripheral blood lineage analysis at 22 weeks showed no difference in levels of myeloid ($Mac1^{+}Gr1^{+}$), T cell ($CD3^{+}$), or B cell ($B220^{+}$) levels between the two groups (Figure 7C). Thus, both shRNA-mediated and germline loss of *Itgb3* does not impair normal HSPC engraftment, reconstitution, or differentiation.

Given the dramatic growth inhibitory effect of *Itgb3* shRNAs on leukemia cells *in vivo*, we examined the effect of expressing the MLL-AF9 fusion gene in $Itgb3^{-/-}$ cells. We hypothesized that if *Itgb3* is required for leukemia growth, there should be an impairment in leukemia formation of bone marrow from $Itgb3^{-/-}$ mice carrying MLL-AF9. We tested this directly in an *in vivo* leukemogenesis latency experiment. $c\text{-}Kit^{+}$ bone marrow cells from either $Itgb3^{-/-}$ or littermate control $Itgb3^{+/+}$ mice were transduced with pMSCV-MLL-AF9-IRES-GFP retrovirus and transplanted into lethally irradiated wild-type recipient mice (Figure S7D). We found that mice transplanted with transduced $Itgb3^{-/-}$ bone marrow lived significantly longer than those transplanted with the transduced $Itgb3^{+/+}$ bone marrow (Figure 7D). The striking differences observed between malignant and normal

hematopoiesis using both RNAi and germline knockout studies demonstrate the selective importance of *Itgb3* in leukemia and potential as a therapeutic target.

DISCUSSION

Integrins play a role in multiple cellular processes relevant to cancer, including homing, adhesion, motility, proliferation, and apoptosis (Desgrosellier and Cheresh, 2010; Guo and Giancotti, 2004). We identified *Itgb3* as a gene that is selectively functionally essential for murine and human leukemia cells, relative to normal HSPCs, using a series of *in vivo* genetic screens. Loss of *Itgb3* in leukemia cells by both RNAi and germline deletion impaired leukemia cell growth while having no effect on normal HSPCs *in vivo*. Leukemia cells carrying an *Itgb3* shRNA were depleted by > 50 fold in less than 2-weeks whereas normal HSPCs carrying the same shRNA were unaffected after 24 weeks. Leukemia cells from both the M9 leukemia model and primary leukemia samples carrying *ITGB3* shRNAs also exhibited impaired growth *in vivo*. Moreover, we found leukemia cells to be dependent on *Itgav*, the heterodimeric partner of *Itgb3; Syk*, a kinase downstream of *Itgb3*; and genes encoding other interacting proteins or downstream signaling molecules. Knockdown of *Itgb3* impaired homing of primary leukemia cells and induced myeloid differentiation in our murine model and in two human leukemia cell line models in addition to causing decreased levels of phosphorylated SYK in MLL and non-MLL contexts. In contrast, genetic loss of *Itgb3* in normal hematopoietic cells did not impair stem or progenitor cell function or differentiation in the primary transplant model, highlighting a potential mechanism by which *Itgb3* inhibition selectively impairs leukemia cell growth.

We identified multiple members of the Itgb3 signaling pathway that are also essential for leukemia, including *Syk*, *Vav1*, *Rac2*, *Rhoa*, *Ptk2b*, *Pak6*, and *CD47*. Knockdown of *Syk* or small molecule Syk inhibition impaired leukemia cell growth, activated SYK rescued the effects of *Itgb3* knockdown, and *ITGB3* downregulation decreased p-SYK levels. Of note, small molecule SYK inhibitors have shown activity against AML in xenotransplant models, again highlighting the biological and therapeutic connection between Itgb3 and Syk (Hahn et al., 2009).

The identification of *Itgb3* highlights the utility of *in vivo* genetic screens to discover potential therapeutic targets in a physiologic microenvironment. In addition, we employed pooled *in vivo* shRNA screens for the genetic dissection of human cancer cells in a xenotransplantation model and the systematic examination of the members of a biological pathway. The screens are quantitative, using massively parallel sequencing with thousands of reads per shRNA, and are highly robust, with the same shRNAs scoring across replicates, tissues (bone marrow and spleen), and different pooled screens.

Our findings highlight a critical axis for the biology and treatment of AML. The identification of the leukemia-selective dependence on *Itgb3*, confirmation of *ITGB3* importance in human disease, molecular dissection of *Itgb3* signaling, and understanding of the cellular programs influenced by *Itgb3* highlights the significance of this pathway for therapeutic interventions. Our studies also provide a paradigm to incorporate physiologically relevant screening strategies to further drive biological and drug discovery efforts for this and other highly aggressive malignancies.

EXPERIMENTAL PROCEDURES

Lentiviral and retroviral production, infection, and screening

Lentivirally-expressed shRNAs in the pLKO.1 backbone vector were obtained from the RNAi Consortium at the Broad Institute. Production of lentiviral supernatants was

performed as described previously (Luo et al., 2008). Pooled lentivirus for screening was generated either by pooling equal amounts of the lentiviral backbone vector DNA prior to lentiviral production, or by pooling titered virus for each individual shRNA to ensure equal representation of each shRNA. In the murine validation screen, each subpool of 55 shRNAs was introduced into 1 million cells, yielding an average of about 4500 cell infected with each shRNA. See Supplementary Experimental Procedures for further details of DNA harvest and sequencing for shRNA representation and a list of shRNAs used in follow up studies.

Mouse Maintenance and Murine Studies

All mouse experiments were conducted under IUCAC-approved animal protocols at Children's Hospital Boston. Mouse strains used in this study include C57BL/6 (Taconic), C57BL/6 Actin-dsRed (Jackson Labs), and NOD-SCID/IL2R$\gamma^{-/-}$ (NSG – Jackson Labs). Recipient mice were either sublethally or lethally irradiated (1 × 5.5 Gy [550 rads] or 2 × 5.5 Gy [550 rads], respectively) prior to tail vein transplantation, as noted in the text. Transplanted cells were resuspended in 300 μL Hank's Balanced Salt Solution (Lonza) and loaded in 27½ gauge syringes (Becton Dickinson).

To generate leukemia cells, primary GMPs (Lin^{lo}, Sca-1, c-Kit^{+}, FcγRII^{hi}, CD34^{hi}) were purified from C57BL/6 Actin-dsRed mice using flow cytometry, transduced with pMSCV-MLL-AF9-Neo, and transplanted into lethally irradiated C57BL/6 recipients as previously described (Krivtsov et al., 2006). After disease onset, cells were harvested from the spleens and transplanted into sublethally irradiated secondary recipients. Transplantation of bulk splenocytes from leukemic secondary mice was subsequently repeated twice to generate leukemia cells from quaternary transplant leukemic mouse spleens. See Supplementary Experimental procedures for the details of isolation and infection of normal leukemia cells for screening and follow up studies.

For the *Itgb3* knockout studies, c-Kit^{+} cells from *Itgb3*$^{-/-}$ or littermate control *Itgb3*$^{+/+}$ mice were harvested and transplanted into wild-type recipients with helper bone marrow as described in the Supplemental Experimental Procedures. Chimerism analysis was performed using the CD45.1/2 system.

Human Primary Cell Analysis and shRNA Screen

For all human studies, informed consent was obtained and performed with approval from the University of Rochester Institutional Review Board. Both the human shRNA M9 screen, performed in M9 cells (Barabe et al., 2007), and the studies in primary, cryopreserved AML samples were performed in 5 replicates and transplanted into sublethally irradiated NSG mice. Sample isolation was performed in an analogous manner to the murine studies. See Supplemental Procedures for details of culturing, infection, and harvesting protocols.

Cell Cycle, Homing, and Differentiation

Cell cycle analysis of primary leukemia cells was performed three days after lentiviral infection using Hoechst 33342 dye. For the *in vivo* homing analysis, cells were isolated either 16 hours after transplant from the bone marrow and compared to input or analyzed after 24 hours using *in vivo* 2-photon microscopy as previously described (Fujisaki et al., 2011) – see Supplementary Experimental procedures for more information. Differentiation analysis of murine leukemia, HL-60, and U937 was done with Giemsa-Wright staining and flow cytometry using c-Kit, CD11, and CD14 antibodies.

Gene Expression Studies

RNA was isolated using either Trizol or an RNeasy kit (Qiagen) and cDNA was synthesized with SuperScript III (Invitrogen). Gene expression analysis was performed on either the MouseWG-6 v2.0 Expression BeadChip (Illumina) or HT HG-U133A arrays. See Supplemental Experimental Procedures for details of sample preparation.

Data Analysis and Statistics

Kaplan Meyer analysis was performed using Prism 5 (GraphPad) software. In all figures, the mean and standard error of the mean are shown. For the shRNA screens, the raw sequencing data was normalized to the total number of reads for each replicate. The fold change was calculated as the ratio of normalized reads between two time points, divided by the ratio of read counts of control shRNAs between the same time points. A gene was considered a hit in the primary screen, ITGB3 targeting screen, and M9 screen if two shRNAs had greater than a fold change of 10. For the validation screen a depletion of 20-fold and enrichment of 2-fold were used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *In Vivo* RNAi screening in primary mouse and human AML reveals dependence on *Itgb3*
- *Itgb3* depletion impairs leukemia cell growth while sparing normal hematopoiesis
- *Itgb3* loss downregulates leukemia stem cell programs and induces differentiation
- Itgb3 activity in AML is mediated by Syk and amenable to pharmacologic inhibition

Significance

Effective therapy for acute myeloid leukemia (AML) requires the elimination of leukemia cells residing in the bone marrow, a microenvironment that supports the survival of both normal and malignant hematopoietic cells. In a series of in vivo RNA interference screens, we identified molecules that are essential for leukemia cells *in vivo*, including integrin beta 3 (*Itgb3*), its heterodimeric partner integrin alpha v (*Itgav*), and downstream signaling molecules. We found Syk phosphorylation to be an essential mediator of Itgb3 activity in AML. Loss of *Itgb3* or *Syk* induced differentiation and decreased expression of leukemia stem cell gene expression signatures. In contrast, genetic inactivation of *Itgb3* did not impair normal hematopoiesis, highlighting the potential of targeting Itgb3 signaling to treat AML.

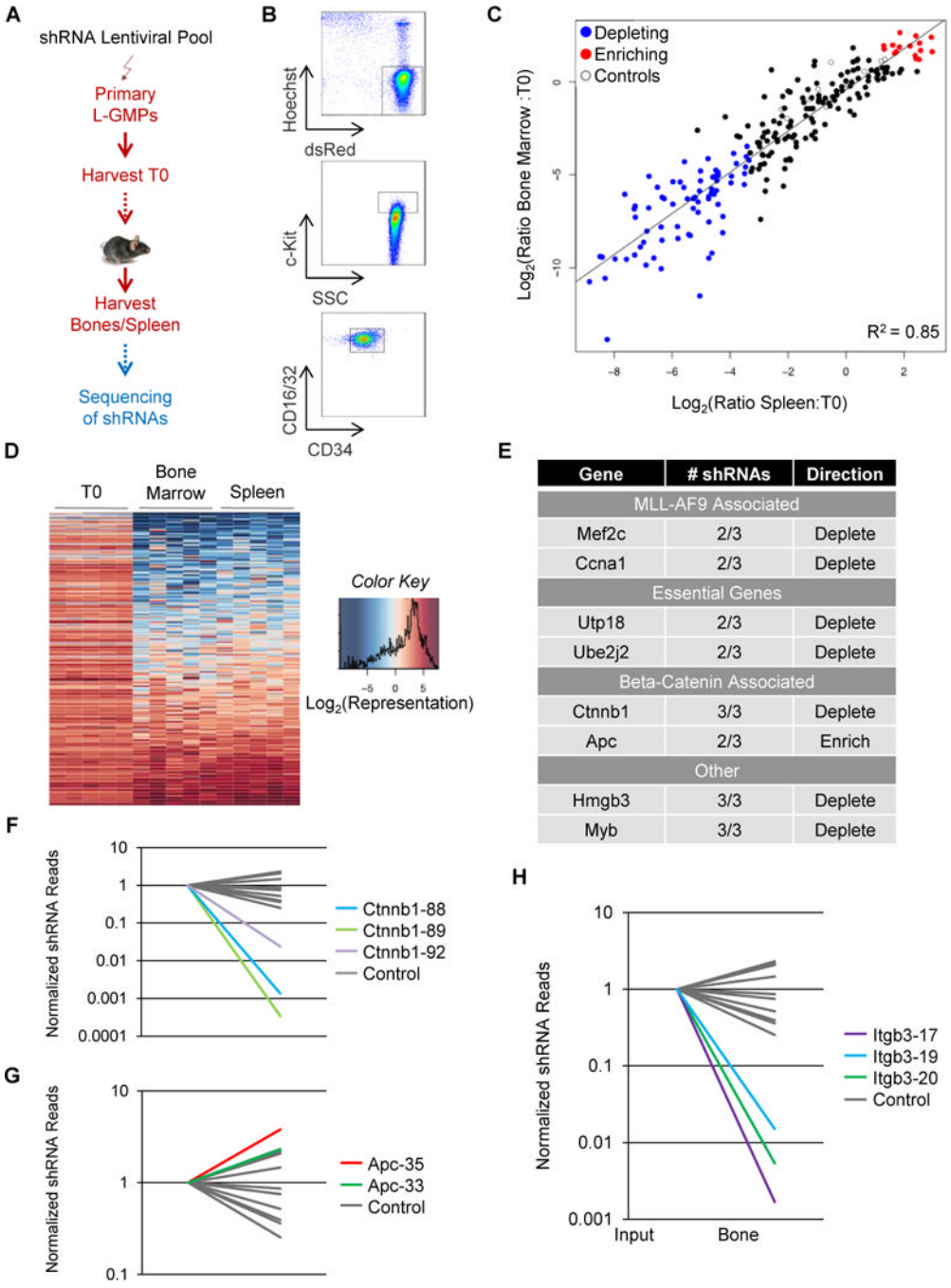

Figure 1. Pooled *In Vivo* shRNA Screening of Primary Leukemic Cells Identifies *Itgb3* as a Mediator of Leukemia Progression

(A) The primary screen (green) was performed using LSCs isolated from quaternary transplant leukemia. The *in vivo* arm was performed in 5 replicate mice, and the *in vitro* arm was performed on the OP9 stromal cell line in 6 replicates. The primary screen hits were retested in 5 separate subpools with 5 replicates per mouse in the validation screen (red). T0 is the time point 24 hours after infection. (B) Primary LSCs (Hoechst^{lo} dsRed^{+} c-Kit^{hi} CD34^{+} FcγRII^{hi}) driven by the MLL-AF9 oncogene used for screening were isolated from the bone marrow of moribund quaternary transplant leukemic mice. (C) A scatterplot of the change in shRNA representation in the validation screen after *in vivo* growth shows high

concordance between the bone marrow and spleen compared to input. For genes highlighted in blue, at least 2 shRNAs depleted by 20-fold in either the bone marrow or spleen. (D) In the heatmap depiction of the validation screen, individual shRNAs (rows) in each replicate (columns) are shown in deep red for the highest number of reads, and in blue for low numbers of reads. (E) Multiple shRNAs targeting positive control genes were depleted by 20-fold in the validation screen. The prevalence of test shRNAs (colored lines) and control shRNAs (gray lines) over time in the validation screen is shown for *Ctnnb1* (F), *Apc* (G), and *Itgb3* (H). See also Figure S1 and Tables S1 and S2.

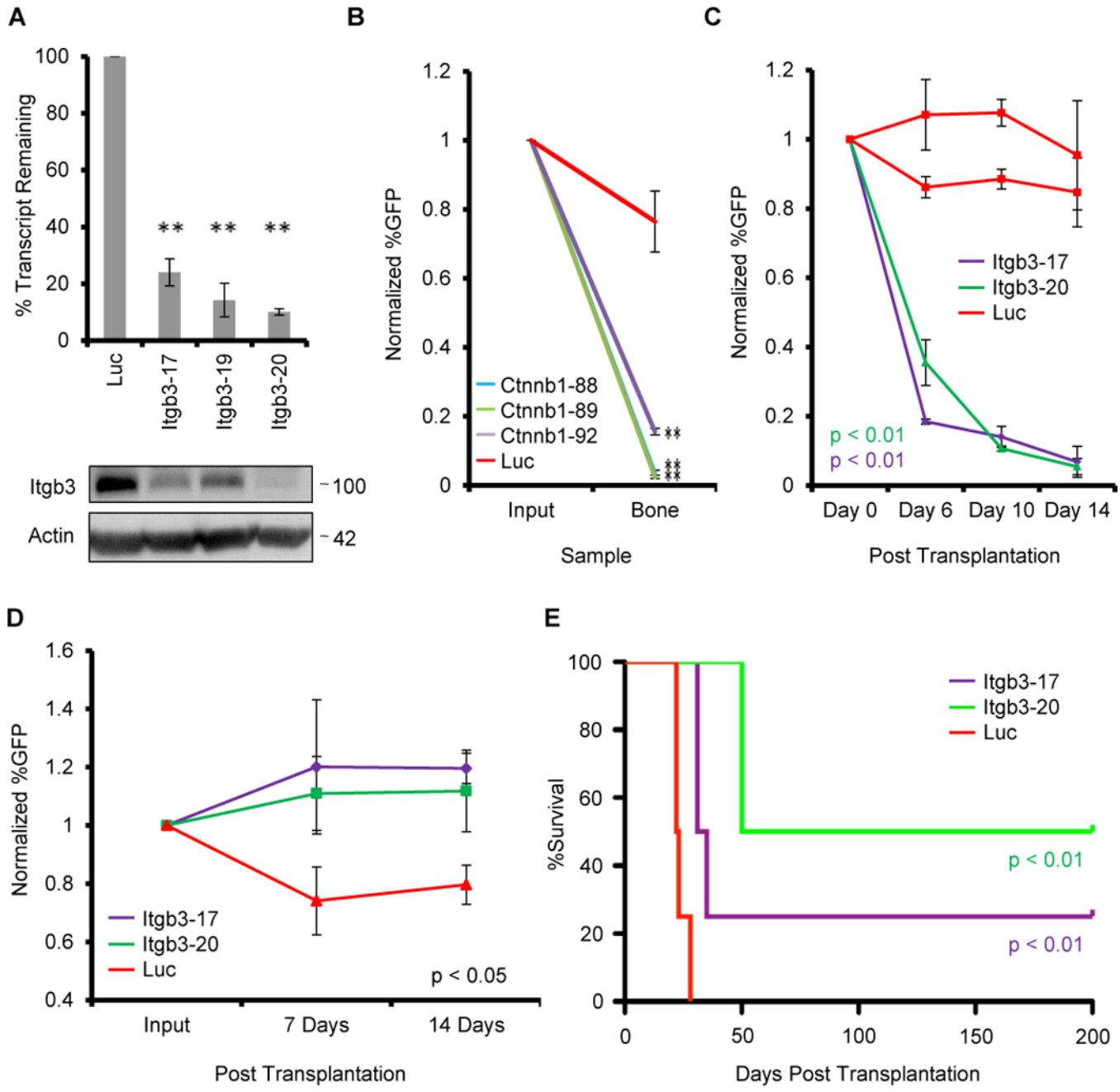

Figure 2. *Itgb3* Knockdown Impairs Leukemia Cell Growth and Survival *In Vivo*

(A) *Itgb3* RNA and protein levels were assessed in Ba/F3 cells after infection with *Itgb3* or control shRNAs. *Ctnnb1* (B), *Itgb3* (C) and control (shLuc) shRNAs were cloned into GFP-expressing lentiviral vectors, transduced into primary leukemias, transplanted, and the percent of GFP^+ cells was assessed before transplant and in the bone marrow after 2 weeks *in vivo* (**p < 0.01). Normalized GFP reflects the ratio of GFP at a given time point to the level immediately after infection. (D) Primary leukemia cells carrying pMIG-$Itgb3_{Rescue}$ (GFP+) were enriched in the bone marrow of sublethally irradiated recipients over 2 weeks in the presence of *Itgb3* but not control shRNAs (p < 0.05, significance determined by two-way ANOVA analysis). (E) 50,000 leukemia cells carrying *Itgb3* or control shRNAs were transplanted into sublethally irradiated mice and monitored for time until death. Mice

transplanted with cells expressing *Itgb3* shRNAs had a significantly prolonged survival, as determined by Kaplan-Meier survival analysis (n = 4 mice per group). Error bars represent +/− S.E.M. See also Figure S2.

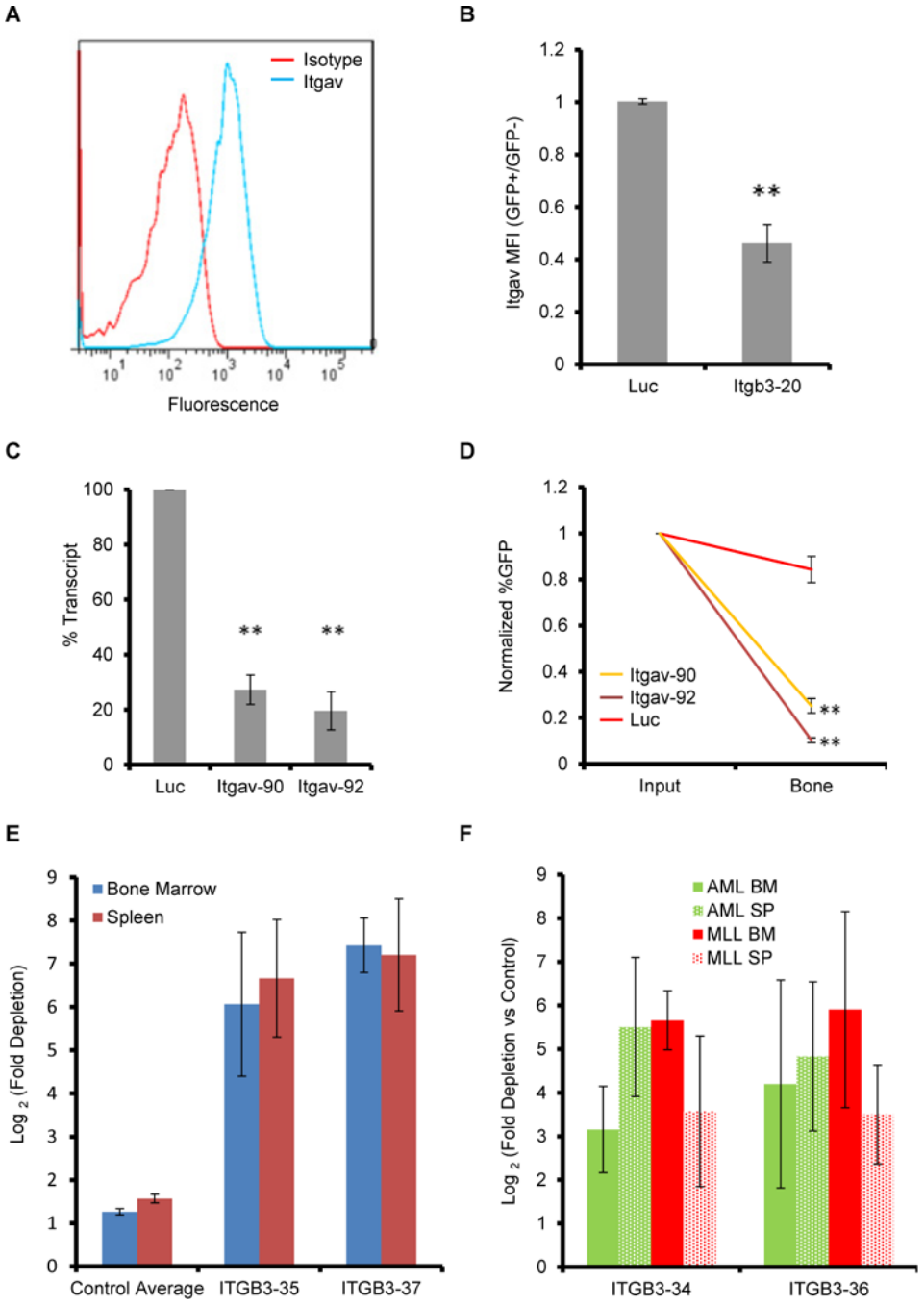

Figure 3. The Itgb3/Itgav Heterodimer is Required for Murine and Human Leukemia Growth *In Vivo*

Cell surface expression of Itgav on unmanipulated primary murine leukemia cells (A) or primary cells infected with *Itgb3*-20 or control shRNAs after 7 days *in vivo* (B) was determined by flow cytometry. *Itgav* shRNAs decrease *Itgav* transcript levels (C) and decrease leukemia cell growth after two weeks *in vivo* as assessed by percent of GFP^+ cells (D). (E) Two *ITGB3* shRNAs were strongly depleted from M9 cells in the bone marrow (blue) and spleen (red) after 3 weeks growth in NSG mice. (F) *ITGB3* shRNAs impair the *in vivo* growth of both cytogentically normal (green) or MLL-rearranged (red) primary human AML cells as assessed by shRNA representation in the bone marrow and spleen of NSG

recipient mice after 6 (MLL-rearranged) or 8 (normal cytogenetics) weeks. (**p < 0.01). Error bars represent +/− S.E.M. See also Figure S3.

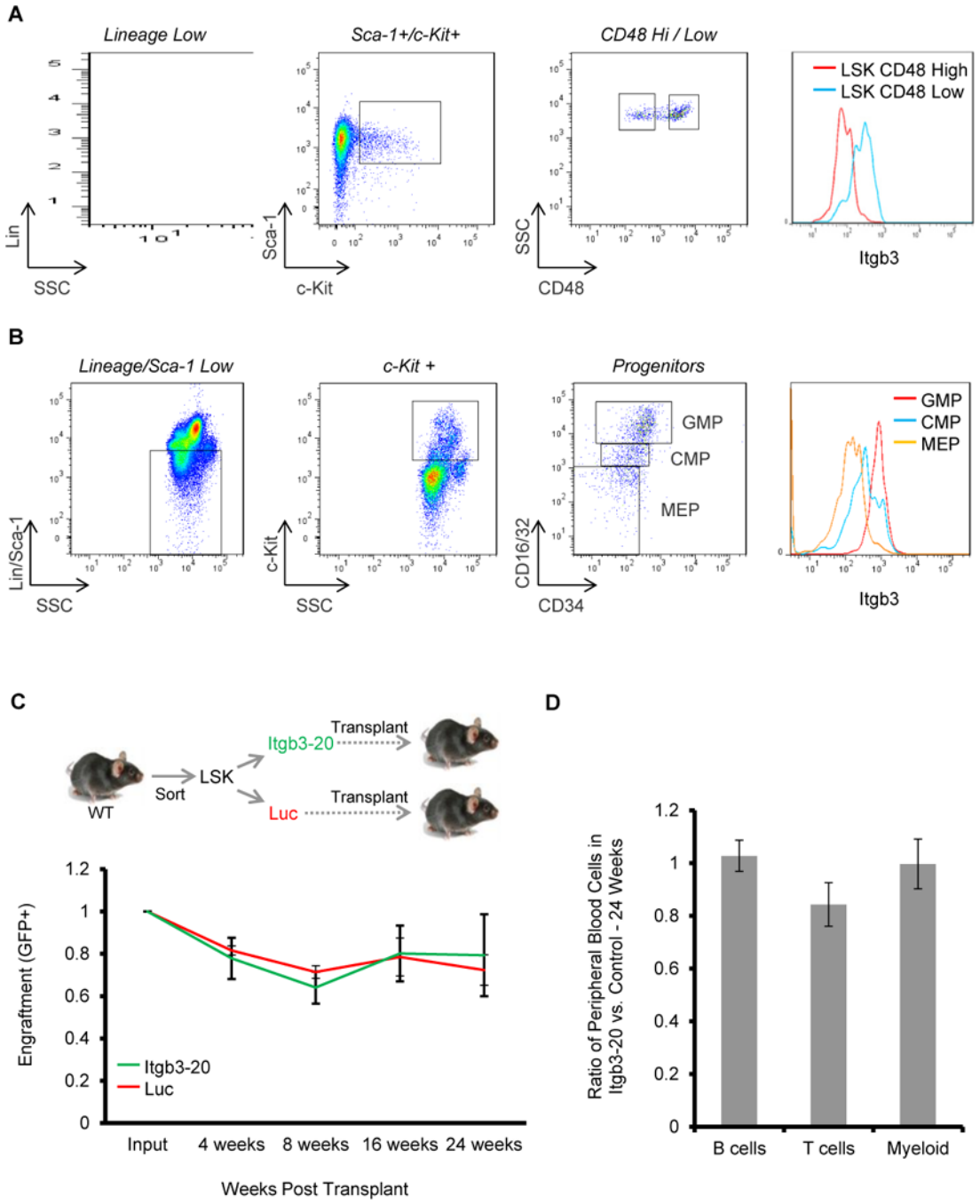

Figure 4. shRNA-Mediated Loss of *Itgb3* Does Not Impair Normal HSPC Function *In Vivo*

Flow cytometric analysis of normal murine LSK cells (A) and progenitor cells (B) shows *Itgb3* expression across hematopoiesis. Representative flow-cytometric plots of gating strategies are shown. (C) 30,000 wild-type LSK cells were infected with *Itgb3*-20 or control shRNAs and transplanted into lethally irradiated recipients, and the percent of GFP^+ cells in the peripheral blood was followed over 24 weeks (n = 6 *Itgb3-20*, n = 5 *Luc*). (D) There is no difference in peripheral blood B, T, or myeloid cells between the *Itgb3* and control shRNA groups. Error bars represent +/− S.E.M. See also Figure S4.

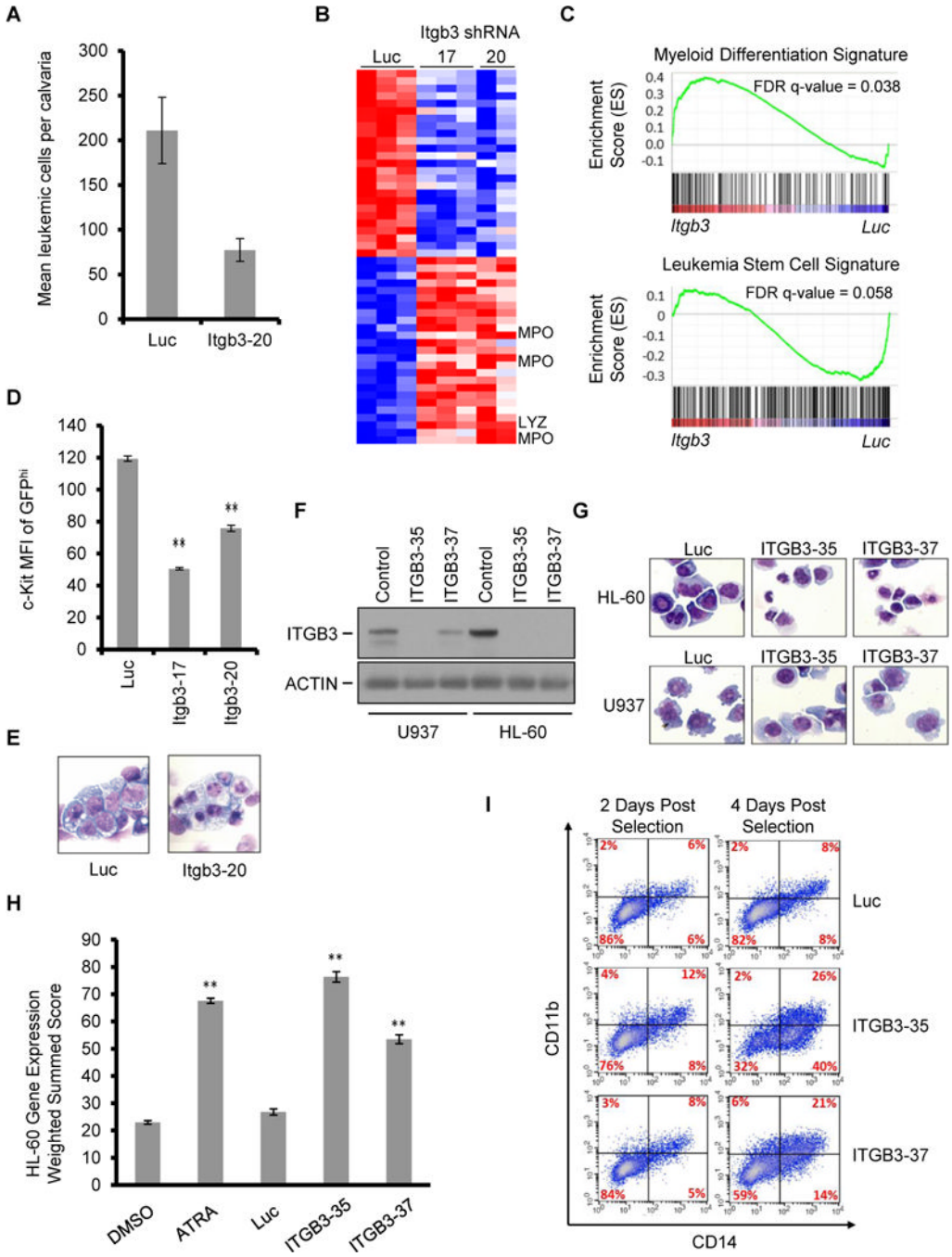

Figure 5. *Itgb3* Knockdown in Primary Leukemia Cells Impairs Homing and Induces Differentiation

(A) Leukemia cells carrying *Itgb3* shRNAs do not home to the bone marrow as efficiently as those carrying control shRNAs, as evidenced by *in vivo* two-photon microscopic analysis of recipient mice calvaria. Shown is the mean number of leukemic cells per mouse calvaria 24 hours after transplantation (n = 3). (B) Heatmap depiction of genes with increased (red) and decreased (blue) expression in primary leukemia cells infected with *Itgb3* relative to controls (MPO – myeloperoxidase, LYZ – lysozyme). (C) Gene set enrichment analysis shows increased expression of a myeloid differentiation signature (Krivtsov et al., 2006) (top) and decreased expression of a leukemia stem cell signature (Somervaille et al., 2009) (bottom) in

leukemia cells with *Itgb3* knockdown relative to controls. Compared to control, leukemia cells carrying *Itgb3* shRNAs (GFP^{hi}) have decreased cell surface c-Kit expression (D) and have morphologic evidence of monocytic differentiation with Wright-Giemsa staining (E) after 3 days. (F) Human *ITGB3* shRNAs (ITGB3-35, ITGB3-37) decrease ITGB3 protein expression in U937 and HL-60 cells relative to a control shRNA (shCT). (G) Wright-Giemsa staining of HL-60 and U937 cells carrying *ITGB3* shRNAs show evidence of differentiation compared to control. *ITGB3* knockdown in HL-60 cells results in increased levels of differentiation as assessed by both the expression of a myeloid gene expression signature, summarized by a single summed score of normalized gene expression (H) and increased cell surface expression of the myeloid differentiation markers CD11b and CD14 (I). (**$p < 0.01$) Error bars represent +/− S.E.M. See also Figure S5 and Table S3.

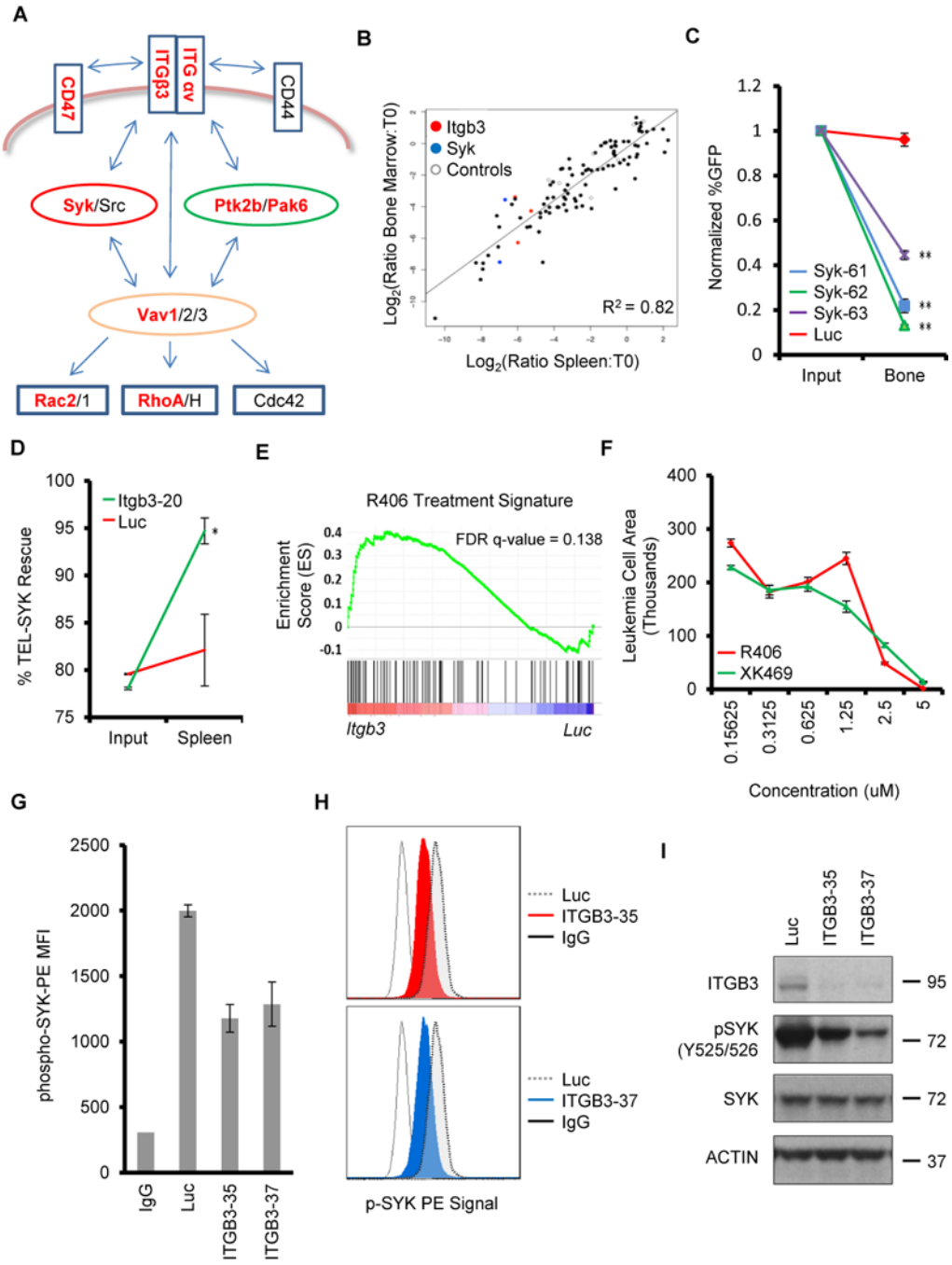

Figure 6. *In Vivo* Itgb3 Pathway shRNA Screen Identifies Syk as a Mediator of Itgb3 Activity

(A) A simplified depiction of the Itgb3/Itgav pathway with hits from the *Itgb3*-targeted screen shown in red. (B) A scatterplot of the change in shRNA representation in the bone marrow and spleen compared to input shows high concordance ($R^2 > 0.8$) and highlights the activity of *Itgb3* 17, 19, and 20 shRNAs (red). (C) *Syk* and control shRNAs, in GFP-expressing lentivirus, were introduced into primary leukemia cells. The percent of GFP^+ cells was assessed before transplant and in the bone marrow after 2 weeks growth *in vivo*. (D) Primary leukemia cells in the spleen carrying pMIG-TEL-SYK (GFP^+) increase over time in the presence of *Itgb3-20* but not control shRNAs, reflecting the ability of TEL-SYK to rescue the effect of *Itgb3* knockdown. (E) Gene set enrichment analysis shows increased

expression of a signature generated from leukemia cell lines treated with the SYK inhibitor R406 in leukemia cells with *Itgb3* knockdown relative to controls. (F) Cobblestone area of leukemia cells grown in co-culture on OP9 bone marrow stromal cells was evaluated following seven days of treatment with a topoisomerase II inhibitor (XK469 – green) or a SYK inhibitor (R406 – red). (G) Levels of phosphorylated-SYK in Mono-Mac-6 cells were determined using intracellular flow cytometry after introduction of control or *ITGB3* shRNAs; representative histograms are shown (H). (I) ITGB3, p-SYK, and SYK levels were also assessed using western blot analysis in the Mono-Mac-6 cells after infection with the shRNAs. (*$p < 0.05$, **$p < 0.01$). Error bars represent +/− S.E.M. See also Figure S6.

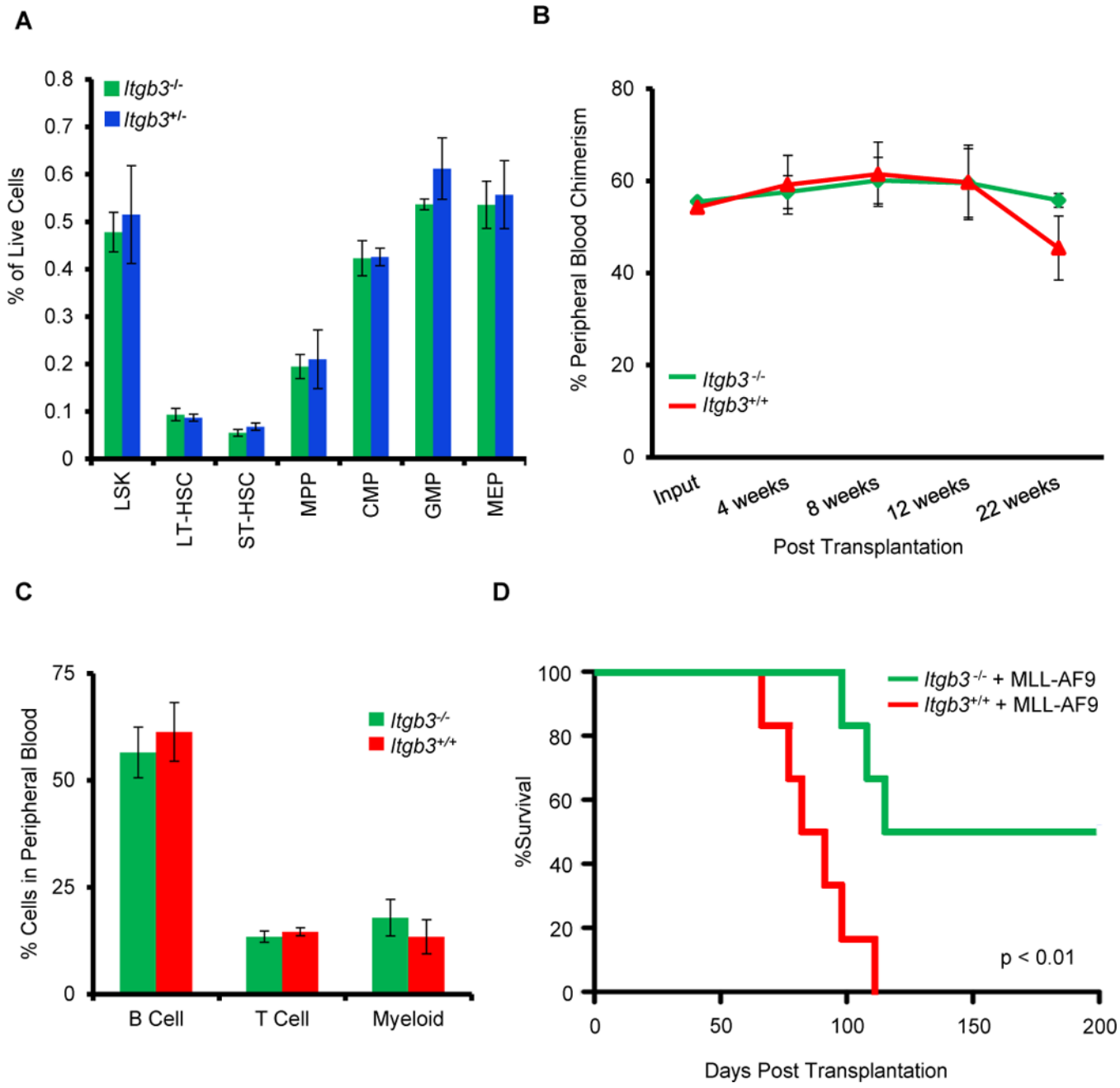

Figure 7. Germline Loss of *Itgb3* Selectively Impairs Leukemogenesis

(A) Bone marrow immunophenotypic analysis reveals no difference in the frequency of different hematopoietic progenitor populations between $Itgb3^{-/-}$ and $Itgb3^{+/-}$ mice. (B) There is no difference between $Itgb3^{-/-}$ and $Itgb3^{+/+}$ hematopoietic stem cell function in long term competitive transplant assays as evidenced by peripheral blood chimerism (n = 6 each group) at 22 weeks. (C) Recipient mice transplanted with bone marrow from either $Itgb3^{-/-}$ or $Itgb3^{+/+}$ mice show no difference in the levels of differentiated cells in the peripheral blood after 22 weeks. (D) Bone marrow from littermate $Itgb3^{-/-}$ (blue) and $Itgb3^{+/+}$ (green) mice were enriched for c-Kit^{+} cells using magnetic beads, infected with pMSCV-MLL-AF9-IRES-GFP and transplanted into lethally irradiated recipient mice. Mice

transplanted with the *Itgb3*$^{-/-}$ transduced cells lived significantly longer. Error bars represent +/− S.E.M. See also Figure S7.